REMARKS

No claims are added. Claims 1-23 and 26 are canceled. Claims 24 and 28 are amended. Claims 24, 25 and 27-34 are currently pending.

Rejection of claims 23-25, 28-30, 33 and 34 as indefinite

The Examiner rejects claims 23-25, 28-30, 33 and 34 under 35 U.S.C. 112 as indefinite because the previous amendment did not indicate removal of the entire structure AA-CO-NH-CH(CH3)CH2OH in independent claim 28. Applicant believes that the Examiner actually meant that the enter structure "AA-CO-O-C(CH2OH)2" was not removed in the prior amendment. Regardless, Applicant has amended claim 28 to indicate removal of both structures, thus obviating the rejection.

Rejection of claims 28-30, 33 and 34 as anticipated

The Examiner also rejects claims 28-30, 33 and 34 under 35 U.S.C. 102(b) as anticipated by a reference to Abadji et al. (R-Methanandamide: A Chiral Novel Anandamide Possessing Higher Potency and Metabolic Stability, J. of Medicinal Chem., 1994, Vol. 37, no. 12, pp. 1889-1893).

Accordingly, Applicant has amended independent claim 28 by removing the compound AA-CO-NH-CH(CH3)CH2OH and respectfully requests that the Examiner withdraw his rejection.

Rejection of claims 25, 27, 28 and 31-34 as anticipated

The Examiner rejects claims 25, 27, 28 and 31-34 under 35 U.S.C. 102(b) as anticipated by a reference to Calignano et al. (Potentiation of anandamide hypotension by the transport inhibitor, AM404, European J. of Pharm., 1997, vol. 337, pp. R1-R2).

As argued in the Response to Office Action filed on August 15, 2006 and *accepted by the Examiner in the Office Action of November 22, 2006*, Calignano

is NOT prior art to the present Application. Regardless, Applicant reiterates his remarks from the August 15, 2006 Response as follows:

- **U.S. Provisional Patent Application No. 60/088,568 supports the use of AM404**

The present Application properly claims priority from U.S. Provisional Patent Application No. 60/088,568. The '568 application discloses the structure of compound AM404 and its use as an anandamide transport inhibitor. The '568 application also provides guidance on where to find synthesis information for compound AM404 (see page 9).

- **Calignano is not prior art under 35 U.S.C. 102(b)**

The present Application properly claims priority from the '568 provisional application filed June 9, 1998. Consequently, the proper effective filing date for the present Application is June 9, 1998 and the proper 102(b) bar date is June 9, 1997.

Calignano was published August 21, 1997. Consequently, Calignano is not prior art under 35 U.S.C. 102(b). At best, Calignano may be prior art under 35 U.S.C. 102(a).

- **A 35 U.S.C. 102(a) reference may be overcome by submission of a declaration under 37 CFR 1.131**

Rejections under 35 U.S.C. 102(a) can be overcome by submitting an affidavit or declaration under 37 CFR 1.131 showing prior invention (see MPEP § 706.02(b)). Enclosed herewith are copies of the Declaration of Alexandros Makriyannis Under 37 CFR 1.131 and Exhibits A-E, as originally submitted with the Response to Office Action of August 15, 2006.

As attested to in the Declaration, Applicant's invention of the recited subject matter predates the publication date of the Calignano reference.

Consequently, Calignano is not prior art under 35 U.S.C. 102(a) and Applicant respectfully requests that the Examiner withdraw the rejection of claims 25, 27, 28 and 31-34 as anticipated by Calignano.

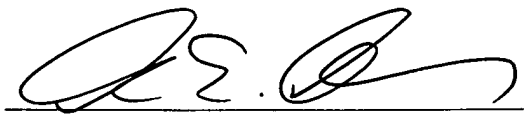
Rejection of claims 23 and 24 as obvious

The Examiner rejects claims 23 and 24 under 35 U.S.C. 103(a) as obvious in view of Abadji. Claim 23 is canceled, thus obviating its rejection. Additionally, claim 24 is amended to remove the stereoisomers of the compound disclosed by Abadji. Consequently, claim 24 is allowable.

For the reasons stated herein, the pending claims are not anticipated or obvious. Applicant respectfully requests that the Examiner withdraw his rejections and pass the pending claims to issue.

Respectfully submitted,

ALEXANDROS MAKRIYANNIS et al.

By: 

Alexander E. Andrews
Registration No. 62,205
Alix, Yale & Ristas, LLP
Attorney for Applicant

Date: 12·01·2008

750 Main Street, Suite 1400
Hartford, CT 06103-2721
(860) 527-9211
Our Ref: UCONAP/141/US

AEA:kcs
G:\AYR saved docs\Filing Docs\Uconap\uconap141us\uconap.141.us-OAResponse112008.doc

DECLARATION OF ALEXANDROS MAKRIYANNIS UNDER 37 C.F.R. 1.131

I, Alexandros Makriyannis, hereby declare:

1. I am a co-inventor named in U.S. Patent Application No. 09/328,742. I have reviewed this application.

2. I am a co-author of a printed publication article titled "Functional Role of High-Affinity Anandamide Transport, as Revealed by Selective Inhibition" published in SCIENCE, volume 277, pages 1094 -1097 and dated August 22, 1997. I have reviewed this article.

3. Attached hereto are documents containing facts showing the preparation of N-(4-hydroxyphenyl)arachidonylamide (compound AM404) in the United States before August 1, 1997 . The dates on all documents have been redacted, which dates are prior to August 1, 1997.

4. Also attached hereto are documents containing facts showing that N-(4-hydroxyphenyl)arachidonylamide (compound AM404) was tested in the United States before the August 1, 1997. The dates on all documents have been redacted, which dates are prior to August 1, 1997.

5. Exhibit A is a photocopy of pages of a laboratory notebook illustrating an experiment testing compound AM404 for anandamide uptake in cells. These pages have been labeled with numbers 000001 to 000004 for convenience.

6. Exhibit B is a photocopy of pages of a laboratory notebook illustrating another experiment testing compound AM404 for anandamide uptake in cells. These pages have been labeled with numbers 000005 to 000008 for convenience.

7. Exhibit C is a photocopy of pages of a laboratory notebook illustrating another experiment testing compound AM404 for anandamide uptake in cells. These pages have been labeled with numbers 000009 to 000012 for convenience.

8. Exhibit D is a photocopy of pages of a laboratory notebook illustrating another experiment testing compound AM404 for anandamide uptake in cells. These pages have been labeled with numbers 000013 to 000016 for convenience.

9. Exhibit E is a photocopy of pages of a laboratory notebook illustrating another experiment testing compound AM404 for anandamide uptake in cells. These pages have been labeled with numbers 000017 to 000020 for convenience.

10. Exhibits A-E illustrate that administration of compound AM404 to cells inhibits transport of anandamide in those cells.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

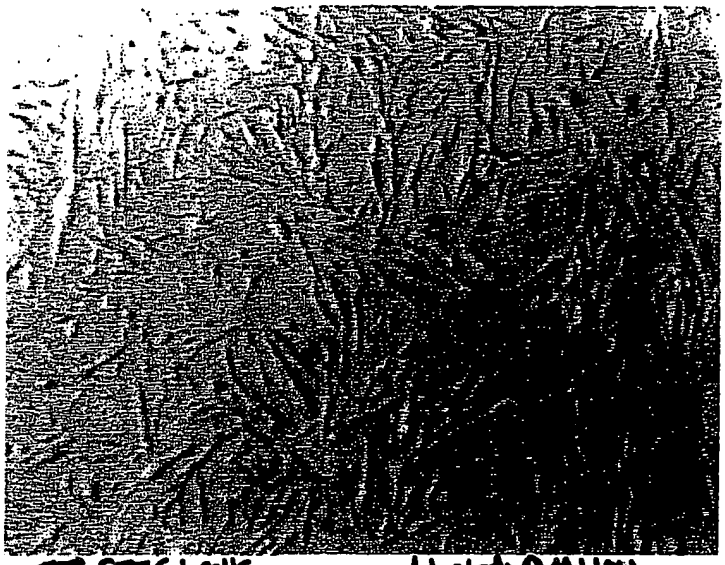
Aug 2, 2006
Date

A. Makriyan
Alexandros Makriyannis

EXHIBIT A

Used CCF-STTG1 cells for an uptake experiment following the uptake protocol 1:00 & utilizing the multichannel pipettor.

Note: For this experiment we are lowering the [ ] of unlabelled anandamide from 100nM to 30nM.



CCF-STTG1 cells cmpd tested: AM404
Plate 317, assay1

Compound tested: AM404
cells 5 days in culture since replating (5th replating)

		30nM unlabelled anandamide + 0.45nM [3H] anandamide			
CCF-STTG1	295-297	"	+	"	+ 0.1% DMSO
	298-300	"	+	"	+ 0.03 μM AM404
	301-303	"	+	"	+ 0.1 μM AM404
	304-306	"	+	"	+ 0.3 μM AM404
	307-309	"	+	"	+ 1.0 μM AM404
	310-312	"	+	"	+ 3.0 μM AM404
	313-315	"	+	"	+ 10.0 μM AM404
	316-318	"	+	"	+ 30.0 μM AM404

Reincubation (w/ the same drug [ ]'s as incubation) was carried out for 13 min. Incubation for 4 minutes. Cells were detached from the plates by sonicating in a water bath for 30 seconds.

CENTCOM : 9 3H180

OCOL : 9 3H180
:
: 19:55
: DL:\P09AS237.TXT
: P09AS237

S113

own samples:

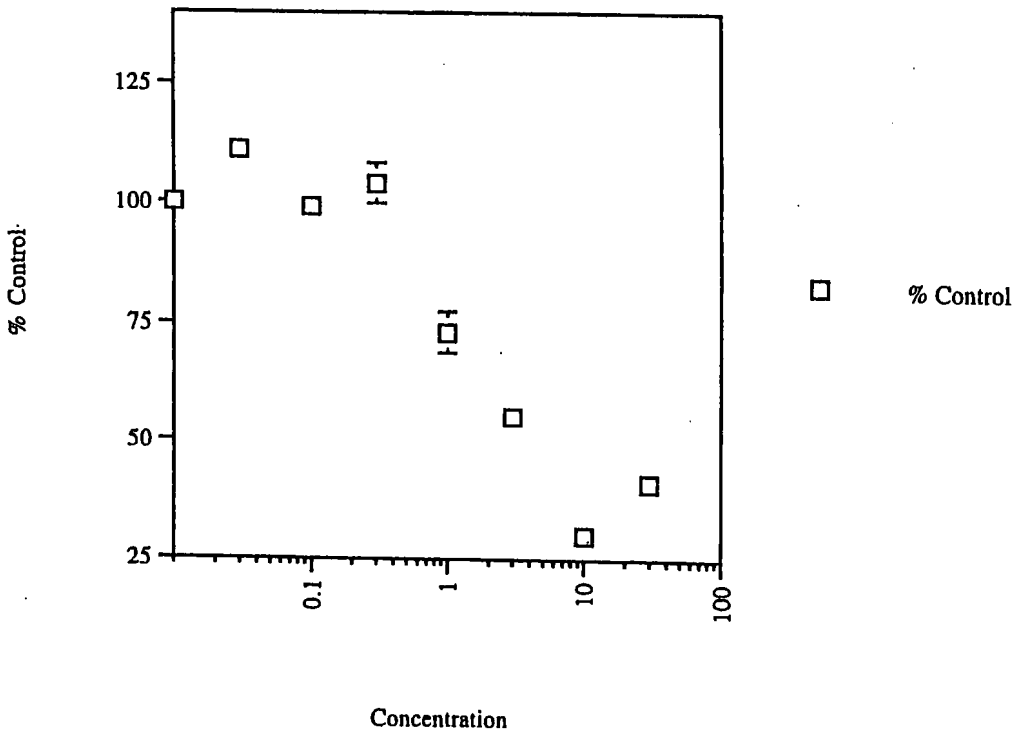
CTime	H3 DPM	
180	43534.0	AM404 + 30um Anand.
180	43183.5	
180	38454.9	control 0
180	49833.2	
180	48430.1	10-0
180	40831.3	
180	5483.6	control
180	5477.5	+ cells
180	5340.3	
180	4778.5	1.0 +
180	4485.8	cells
180	3906.9	

CTime	H3 DPM	
180	43991.7	0.03-0
180	45959.9	
180	39007.3	
180	48100.1	3.0 - 0
180	45546.3	
180	41879.4	
180	6219.1	
181	6460.1	0.03
180	6008.2	+ cells
180	3066.4	
180	3423.2	3.0
180	3165.1	+ cells

CTime	H3 DPM	
180	50545.1	0.1 - c
180	46934.7	
180	39732.6	
180	52023.0	
180	51565.7	10.0 - 0
180	49244.0	
180	6070.1	0.1
180	5914.9	+ cells
180	5783.6	
180	2007.6	10.0
180	2141.6	+ cells
180	1804.4	

CTime	H3 DPM	
180	36614.3	0.3 - 0
180	34329.6	
180	30793.7	
180	54859.6	30.0 - 0
180	59524.3	
180	52615.8	
180	4937.0	
180	4537.3	0.3
180	4287.3	+ cells
180	2935.0	
180	2918.2	30.0
180	2949.9	+ cells

ST-CCF-STTG19705-13AM404



Title	CCF-STTG1-Uptake & Inhibition with AM404												
Column ID	A	B	C	D	E	F	G	H	I	J	K	L	M
Column Title	Control In	0.03 In	0.1 In	0.3 In	1.0 In	3.0 In	10.0 In	30.0 In	Control c	0.03 c	0.1 c	0.3 c	1.0 c
Raw or Mean	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
Mean	41724	42986	45738	33913	46365	45175	50944	55667	5433.33	6229.00	5923.00	4587.00	4390.67
SD	2836.73	3583.93	5504.55	2932.36	4843.37	3127.05	1490.16	3523.94	80.93	226.17	143.17	327.87	443.75
Sample size	3	3	3	3	3	3	3	3	3	3	3	3	3
SEM	1637.79	2069.18	3178.06	1693.00	2796.32	1805.40	860.34	2034.55	46.72	130.58	82.66	189.30	256.20
95% CI min	34677	34083	32063	26628	34332	37406	47242	46912	5232.28	5667.13	5567.32	3772.46	3288.25
95% CI max	48772	51890	59413	41198	58397	52944	54646	64421	5634.39	6790.87	6278.68	5401.54	5493.09
Minimum	38455	39007	39733	30794	40831	41879	49244	52616	5340.00	6008.00	5784.00	4287.00	3907.00
Maximum	43534	45960	50545	36614	49833	48100	52023	59524	5484.00	6460.00	6070.00	4937.00	4779.00

N	O	P
3.0 c	10.0 c	30.0 c
Raw Data	Raw Data	Raw Data
3218.00	1984.67	2934.33
184.31	170.20	16.01
3	3	3
106.41	98.27	9.24
2760.12	1561.82	2894.56
3675.88	2407.51	2974.11
3066.00	1804.00	2918.00
3423.00	2142.00	2950.00

ST-CCF-STTG19705-13AM404

	1	2	3	4	5	6	7	8	9
	Concentration	Initial	In the cell	SEM	Corr fac	In the cell c	SEM c	% Control	SEM %
1	0.01	41724	5433	47	1	5433	47	100	0.87
2	0.03	42986	6229	131	0.971	6048.359	127.201	111	2.34
3	0.1	45738	5923	83	0.912	5401.776	75.696	99	1.4
4	0.3	33913	4587	189	1.23	5642.01	232.47	104	4.27
5	1	46365	4391	256	0.9	3951.9	230.4	73	4.23
6	3	45175	3218	106	0.924	2973.432	97.944	55	1.8
7	10	50944	1985	98	0.819	1625.715	80.262	30	1.47
8	30	55667	2024						

EXHIBIT B

Used CCF-STTG1 cells for an uptake experiment following the uptake protocol 1.00

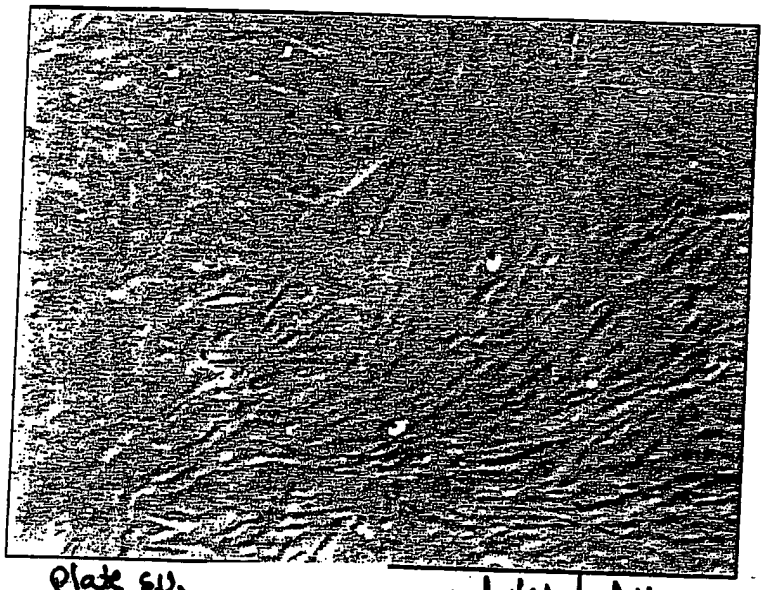


Plate sv. assay

cmpd tested: AM404

cmpd tested: AM404

Cells in culture 7 days since replating.

CCF-STTG1

	30nM unlabelled anandamide + 0.45nM [^{3}H] anandamide	+ ...	
559-561	"	+ "	+ 0.1% DMSO
562-564	"	+ "	+ 0.03 μM
565-567	"	+ "	+ 0.1 μM
568-570	"	+ "	+ 0.3 μM
571-573	"	+ "	+ 1.0 μM
574-576	"	+ "	+ 3.0 μM
577-579	"	+ "	+ 10.0 μM
580-582	"	+ "	+ 30.0 μM

Pre-incubation time: 13 min.
incubation time: 4 min.

ROTOCOL : ? 3H180
ATE : 4/4/94
IME : 23:59
ILE : DL:\P09AS258.TXT
D : P09AS258

nknown samples:

Pos	CTime	H3 DPM
1	180	55543.5
2	180	57370.6
3	180	55113.3
4	180	61061.3
5	180	60186.4
6	180	57045.1
13	180	8850.9
14	180	8076.5
15	180	7544.5
16	180	7241.0
17	180	7454.6
18	180	5743.9

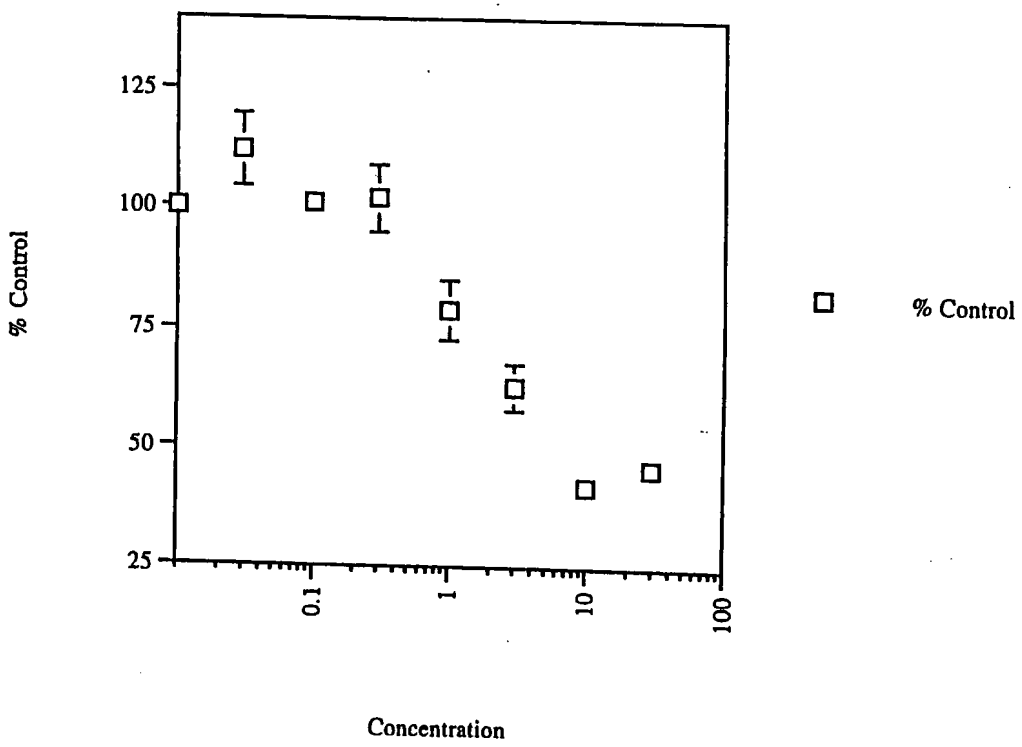
os	CTime	H3 DPM
19	180	58985.6
20	180	58077.3
21	180	54300.6
22	180	68716.9
23	180	68450.1
24	180	65347.3
31	180	10193.6
32	180	9625.0
33	180	8063.9
34	180	6390.7
35	180	6947.8
36	180	5361.0

os	CTime	H3 DPM
37	180	62084.9
38	180	60810.4
39	180	55535.1
40	180	66359.7
41	180	65857.5
42	180	64467.0
49	180	8915.9
50	180	9131.1
51	180	8265.8
52	180	4191.8
53	180	4119.1
54	180	3701.9

s	CTime	H3 DPM
5	180	63513.1
6	180	62565.9
7	180	58652.7
8	180	78918.5
9	180	76599.1
0	180	75823.6
1	180	8915.5
2	180	10321.9
	180	8152.8
	180	5029.0
	180	5278.8
	180	5105.7

000006

ST-9706-09CCF-STTG1 AM404



Title								
Column ID	A	B	C	D	E	F	G	H
Column Title	Control in	0.03 in	0.1 in	0.3 in	1.0 in	3.0 in	10.0 in	30.0 in
Raw or Mean	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
Mean	5.6010e+04	5.7121e+04	5.9477e+04	6.1577e+04	5.9431e+04	6.7505e+04	6.5562e+04	7.7114e+04
SD	1198.57	2484.41	3472.60	2576.43	2111.86	1873.36	980.67	1609.93
Sample size	3	3	3	3	3	3	3	3
SEM	692.00	1434.37	2004.91	1487.50	1219.28	1081.58	566.19	929.49
95% CI min	5.3032e+04	5.0949e+04	5.0850e+04	5.5177e+04	5.4184e+04	6.2851e+04	6.3125e+04	7.3114e+04
95% CI max	5.8987e+04	6.3293e+04	6.8104e+04	6.7978e+04	6.4677e+04	7.2159e+04	6.7998e+04	8.1113e+04
Minimum	5.5113e+04	5.4301e+04	5.5535e+04	5.8653e+04	5.7045e+04	6.5347e+04	6.4467e+04	7.5824e+04
Maximum	5.7371e+04	5.8986e+04	6.2085e+04	6.3513e+04	6.1061e+04	6.8717e+04	6.6360e+04	7.8918e+04

I	J	K	L	M	N	O	P
Control c	0.03 c	0.1 c	0.3 c	1.0 c	3.0 c	10.0 c	30.0 c
Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
8157.67	9294.00	8771.00	9130.00	6813.33	6233.33	4004.33	5138.00
656.73	1102.42	450.36	1100.37	932.23	805.16	264.36	128.04
3	3	3	3	3	3	3	3
379.16	636.48	260.02	635.30	538.22	464.86	152.63	73.92
6526.14	6555.21	7652.15	6396.31	4497.36	4233.04	3347.57	4819.92
9789.20	1.2033e+04	9889.85	1.1864e+04	9129.31	8233.63	4661.09	5456.08
7545.00	8064.00	8266.00	8153.00	5744.00	5361.00	3702.00	5029.00
8851.00	1.0193e+04	9131.00	1.0322e+04	7455.00	6948.00	4192.00	5279.00

ST-9706-09CCF-STTG1 AM404

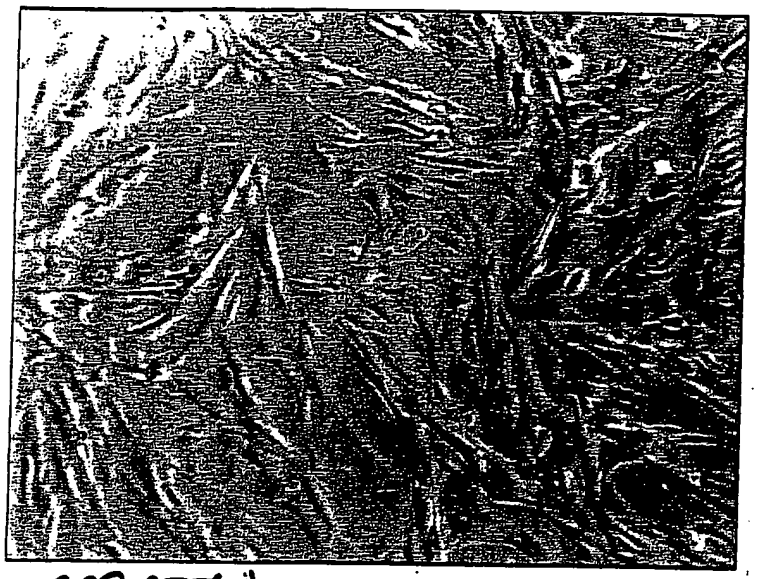
9:00

	1	2	3	4	5	6	7	8	9
	Concentration	Initial	In the cell	SEM	Corr fac	In the cell c	SEM c	% Control	SEM %
1	0.01	56010	8158	.79	1	8158	79	100	0.97
2	0.03	57121	9294	637	0.981	9117.414	624.897	112	7.66
3	0.1	59477	8771	260	0.942	8262.282	244.92	101	3
4	0.3	61577	9130	635	0.91	8308.3	577.85	102	7.09
5	1	59431	6813	538	0.942	6417.846	506.796	79	6.21
6	3	67505	6233	465	0.83	5173.39	385.95	63	4.73
7	10	65562	4004	153					

800058

EXHIBIT C

Used CCF-STTG1 cells for an uptake experiment following the uptake protocol 1.00.



CCF-STTG1 AM404
S.U.
assay

Cmpd tested: AM 404

Cells in culture 4 days since replating.

CCF-STTG1	30 μM unlabelled anandamide + 0.45 nM [3H] anandamide	
655-657	"	+ 0.1% DMSO
658-660	"	+ 0.03 μM AM404
661-663	"	+ 0.1 μM AM404
664-666	"	+ 0.3 μM AM404
667-669	"	+ 1.0 μM AM404
670-672	"	+ 3.0 μM AM404
673-675	"	+ 10.0 μM AM404
676-678	"	+ 30.0 μM AM404

Preincubation time: 8 min.
Incubation time: 4 min.

Cells detached by sonicating for 60 seconds.

ROTOCOL : P 3H15E

ATE :

IME : 23:18

ILE : DC:\P09AS263.TXT

D : P09AS263

nknown samples:

Pos	CTime	H3 DPM
1	180	50021.8
2	180	49601.1
3	180	44804.9
4	180	51277.0
5	180	51767.9
6	180	47349.4
13	180	5396.6
14	180	6893.3
15	180	5364.8
16	180	5036.1
17	180	5458.8
18	180	4969.0

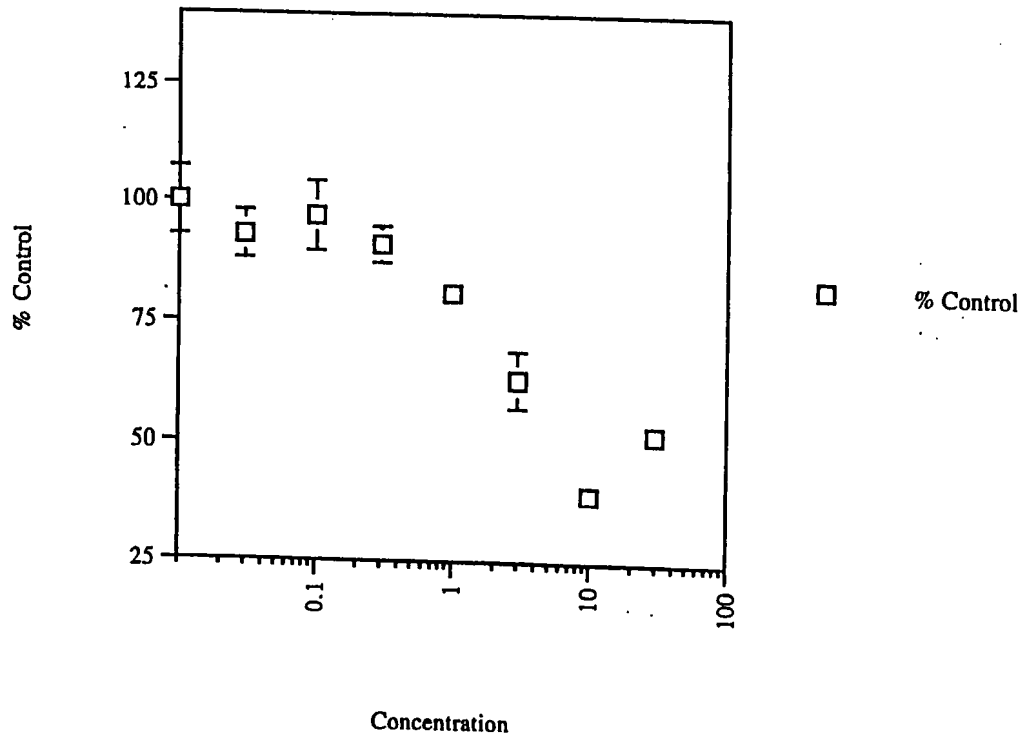
Pos	CTime	H3 DPM
19	180	47786.2
20	180	46721.3
21	180	42048.3
22	180	66515.6
23	180	64736.1
24	180	62094.4
31	180	4836.6
32	180	5821.5
33	180	5350.4
34	180	5527.9
35	180	5757.2
36	180	4231.6

Pos	CTime	H3 DPM
37	180	53650.6
38	180	53189.2
39	180	48001.0
40	180	58030.7
41	180	57678.8
42	180	56950.2
49	180	5412.7
50	180	7018.7
51	180	6563.7
52	180	2869.6
53	180	2709.0
54	180	2999.2

Pos	CTime	H3 DPM
55	180	45751.5
56	180	46571.1
57	180	43326.7
58	180	58315.8
59	180	56239.6
60	180	55350.4
67	180	4982.7
68	180	5707.9
69	180	5155.4
70	180	3344.2
71	180	3754.2
72	180	3989.2

000010

ST-9706-16CCF-STTG1a AM404



Title	CCI-STTG1 Uptake & Inhibition with										
Column ID	A	B	C	D	E	F	G	H	I	J	K
Column Title	Control In	0.03 In	0.1 In	0.3 In	1.0 In	3.0 In	10.0 In	30.0 In	Control c	0.03 c	0.1 c
Raw or Mean	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
Mean	4.8143e+04	4.5518e+04	5.1614e+04	4.5883e+04	5.0131e+04	4.4449e+04	5.7553e+04	5.6635e+04	6085.33	5336.33	6331.33
SD	2898.16	3053.34	3137.18	2624.47	2422.05	2225.09	551.35	1522.01	755.72	492.64	826.92
Sample size	3	3	3	3	3	3	3	3	3	3	3
SEM	1673.25	1762.85	1811.25	1515.24	1398.37	1284.66	318.32	878.73	436.32	284.43	477.43
95% CI min	4.0943e+04	3.7932e+04	4.3820e+04	3.9363e+04	4.4114e+04	3.8921e+04	5.6184e+04	5.2854e+04	4207.87	4112.44	4276.97
95% CI max	5.5343e+04	5.3103e+04	5.9407e+04	5.2403e+04	5.6149e+04	5.9977e+04	5.8923e+04	6.0417e+04	7962.80	6560.22	8385.69
Minimum	4.4805e+04	4.2046e+04	4.8001e+04	4.3327e+04	4.7349e+04	4.2094e+04	5.6950e+04	5.5350e+04	5397.00	4837.00	5413.00
Maximum	5.0022e+04	4.7786e+04	5.3651e+04	4.8571e+04	5.1768e+04	4.6516e+04	5.8031e+04	5.8316e+04	6894.00	5822.00	7017.00

L	M	N	O	P
0.3 c	1.0 c	3.0 c	10.0 c	30.0 c
Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
5282.00	5154.67	5172.33	2859.00	3729.33
378.82	265.68	822.36	145.26	340.57
3	3	3	3	3
218.71	153.39	474.79	83.86	196.63
4340.89	4494.63	3129.31	2498.13	2883.25
6223.11	5814.71	7215.36	3219.87	4575.41
4983.00	4969.00	4232.00	2709.00	3344.00
5708.00	5459.00	5757.00	2999.00	3990.00

ST-9706-16CCF-STTG1a AM404

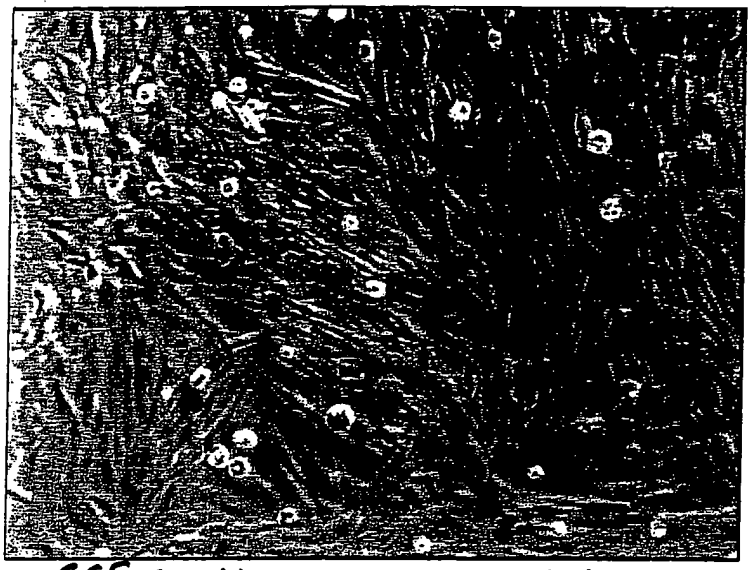
	1	2	3	4	5	6	7	8	9
	Concentration	Initial	In the cell	SEM	Corr fac	In the cell c	SEM c	% Control	SEM %
1	0.01	48143	6085	436	1	6085	436	100	7.17
2	0.03	45518	5336	284	1.058	5645.488	300.472	93	4.93
3	0.1	51614	6331	477	0.932	5900.492	444.564	97	7.31
4	0.3	45883	5282	219	1.049	5540.818	229.731	91	3.78
5	1	50131	5155	153	0.96	4948.8	146.88	81	2.42
6	3	64449	5172	475	0.747	3863.484	354.825	63	5.83
7	10	57553	2859	84	0.836	2390.124	70.224	39	1.15
8	30	56635	3729	197	0.85	3169.65			

8:4

27000

EXHIBIT D

Utilized CCF-STTG1 cells for an uptake experiment following the uptake protocol 1.00



CCF-STTG1 s.u. assay:

Cmpd tested: AM404 (plate reversed)

Cmpd tested: AM404

Cells in culture 5 days since replating.

For an experiment, we reversed the orientation of the plate, to assure that different positioning of the dilutions gave the usual result.

Usual position:

control	1.0
0.03	3.0
0.1	10.0
0.3	30.0

Position this Experiment:

control	1.0
0.03	3.0
0.1	10.0
0.3	30.0

F-STTG1

849-851	30nM unlabelled anandamide + 0.45nM [^{3}H] anandamide			+ 0.1% DMSO	
852-854	"	+	"	+ 0.03 μM	AM404
855-857	"	+	"	+ 0.1 μM	AM404
858-860	"	+	"	+ 0.3 μM	AM404
861-863	"	+	"	+ 1.0 μM	AM404
864-866	"	+	"	+ 3.0 μM	AM404
867-867	"	+	"	+ 10.0 μM	AM404
868-870	"	+	"	+ 30.0 μM	AM404

Pre-incubation time: 13 min.
Incubation time: 4 min.

Cells detached by sonicating for 60 seconds

PROTOCOL : P 3H1CC
DATE :
TIME : 23:20
FILE : DL:\P09AS268.TXT
ID : P09AS268

Unknown samples:

Pos	CTime	H3 DPM
1	180	52885.4
2	180	53120.4
3	180	50669.1
4	180	40572.4
5	180	41113.0
6	180	38202.0
13	180	8638.7
14	180	10742.7
15	180	8976.8
16	180	8857.2
17	180	8703.1
18	180	6984.2

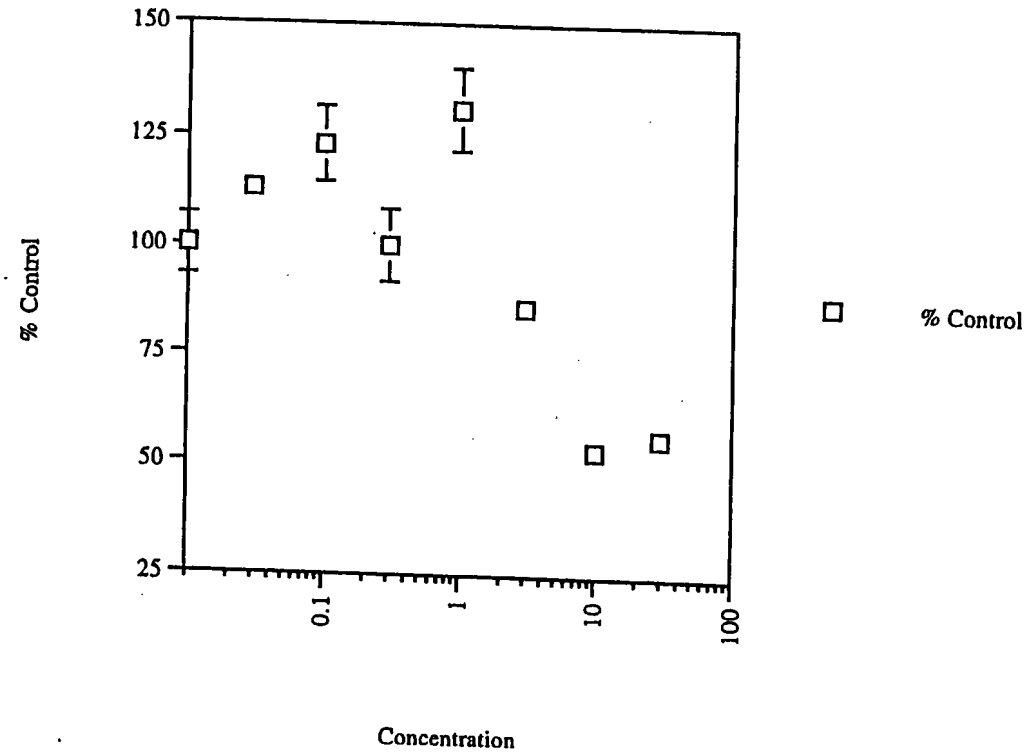
Pos	CTime	H3 DPM
19	180	38553.2
20	180	39000.7
21	180	35904.7
22	180	32065.7
23	180	30294.2
24	180	29954.6
31	180	6467.7
32	180	6820.8
33	180	6553.2
34	180	4223.6
35	180	4269.5
36	180	3875.0

Pos	CTime	H3 DPM
37	180	44371.4
38	180	43487.1
39	180	39536.3
40	180	40173.0
41	180	39243.9
42	180	38363.3
49	180	7367.4
50	180	7776.7
51	180	8212.5
52	180	3594.0
53	180	3209.8
54	180	2919.0

Pos	CTime	H3 DPM
55	180	37022.1
56	180	36587.7
57	180	33428.4
58	180	42599.7
59	180	41136.2
60	180	40664.1
67	180	7681.3
68	180	5987.7
69	180	5077.1
70	180	3430.3
71	180	3822.0
72	180	3552.3

Analyst to Plate reviewed

ST-9706-24CCF-STTG1 AM404



Title	CCI-STTG1 Uptake & Inhibition with										
Column ID	A	B	C	D	E	F	G	H	I	J	K
Column Title	Control In	0.03 In	0.1 In	0.3 In	1.0 In	3.0 In	10.0 In	30.0 In	Control c	0.03 c	0.1 c
Raw or Mean	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
Mean	5.2225e+04	3.7820e+04	4.2458e+04	3.5679e+04	3.9962e+04	3.0772e+04	3.9260e+04	4.1467e+04	8108.67	6613.67	8119.00
SD	1352.36	1673.21	2570.58	1962.90	1548.31	1133.67	905.11	1009.47	974.52	184.63	969.36
Sample size	3	3	3	3	3	3	3	3	3	3	3
SEM	780.79	966.03	1484.12	1133.28	893.91	654.52	522.56	582.82	562.64	106.60	559.66
95% CI min	4.8865e+04	3.3663e+04	3.6072e+04	3.0802e+04	3.6116e+04	2.7955e+04	3.7011e+04	3.8959e+04	5687.64	6154.98	5710.79
95% CI max	5.5584e+04	4.1976e+04	4.8844e+04	4.0555e+04	4.3809e+04	3.3588e+04	4.1509e+04	4.3975e+04	1.0530e+04	7072.36	1.0527e+04
Minimum	5.0669e+04	3.5905e+04	3.9536e+04	3.3426e+04	3.8202e+04	2.9955e+04	3.8363e+04	4.0664e+04	6984.00	6467.00	7367.00
Maximum	5.3120e+04	3.9001e+04	4.4371e+04	3.7022e+04	4.1113e+04	3.2066e+04	4.0173e+04	4.2600e+04	8703.00	6821.00	9213.00

L	M	N	O	P
0.3 c	1.0 c	3.0 c	10.0 c	30.0 c
Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
5532.50	8115.00	4122.67	3241.00	3601.67
644.17	979.73	215.66	338.57	200.48
2	3	3	3	3
455.50	565.65	124.51	195.47	115.75
-255.08	5681.01	3586.89	2399.89	3103.61
1.1320e+04	1.0549e+04	4658.45	4082.11	4099.73
5077.00	6984.00	3875.00	2919.00	3430.00
5988.00	8703.00	4269.00	3594.00	3822.00

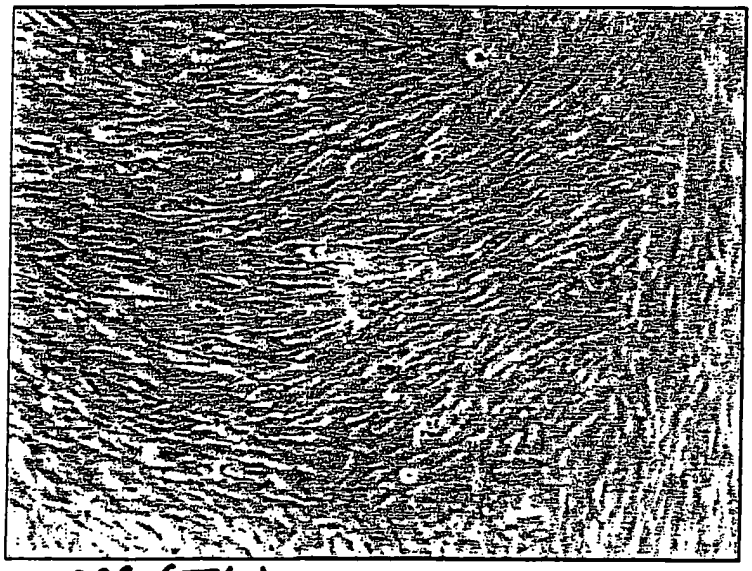
ST-9706-24CCF-STTG1 AM404

	1	2	3	4	5	6	7	8	9
	Concentration	Initial	In the cell	SEM	Corr fac	In the cell c	SEM c	% Control	SEM %
1	0.01	52225	8109	563	1	8109	563	100	6.94
2	0.03	37820	6614	107	1.381	9133.934	147.767	113	1.825
3	0.1	42458	8119	560	1.23	9986.37	688.8	123	8.5
4	0.3	35679	5533	455	1.464	8100.312	666.12	100	8.21
5	1	39962	8115	566	1.307	10606.305	739.762	131	9.13
6	3	30772	4123	125	1.697	6996.731	212.125	86	2.61
7	10	39260	3241	195	1.33	4310.53	259.35	53	3.19
8	30	41467	3602	116	1.259	4534.918	146.044		

8:

EXHIBIT E

Used CCF-STTG1 cells for an uptake experiment following the uptake protocol 1:00:



CCF-STTG1 S.U. assay Cmpd tested: AM404

Cmpd tested: AM404

Cells in culture 6 days since replating.

CCF-STTG1

991-993	30nM unlabelled anandamide + 0.45 nM [3H] anandamide	+	0.1% DMSO		
994-996	"	+	"	+	0.1 µM AM404
997-999	"	+	"	+	0.3 µM AM404
1000-1002	"	+	"	+	1.0 µM AM404
1003-1005	"	+	"	+	3.0 µM AM404
1006-1008	"	+	"	+	10.0 µM AM404
1009-1011	"	+	"	+	30.0 µM AM404
1012-1014	"	+	"	+	100.0 µM AM404

Pre-incubation time: 13 min
Incubation time: 4 min

Cells detached by sonicating 60 seconds

Unknown samples:

CCF-5??
AM484

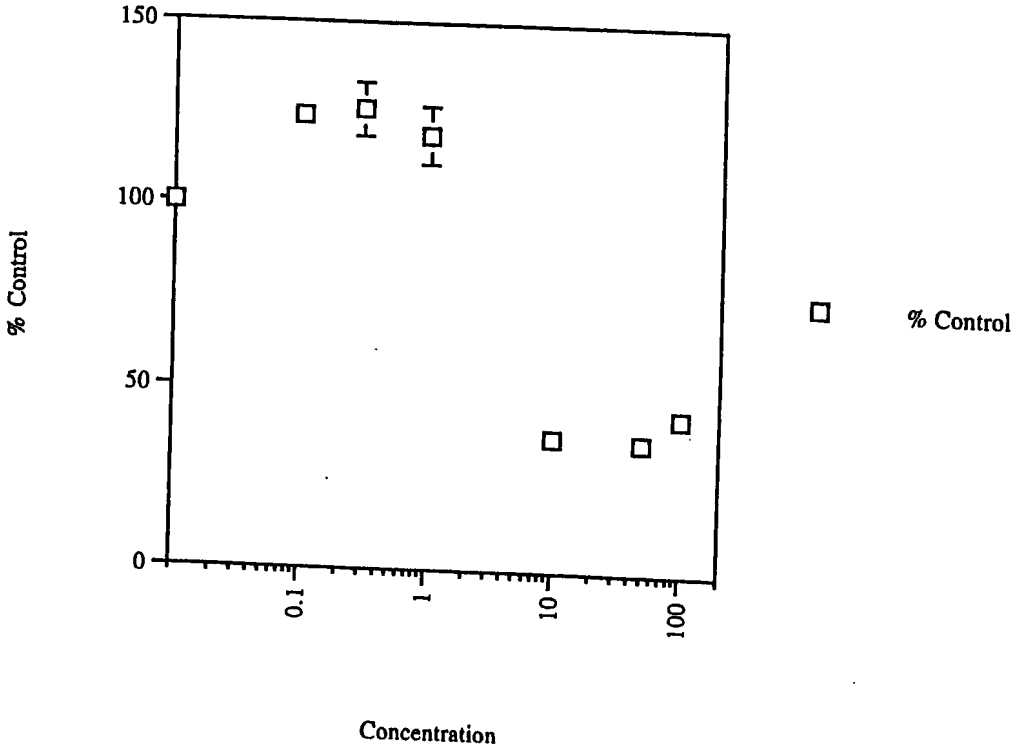
Pos	CTime	HJ DPM
1	180	40563.8
2	180	37698..
3	180	34045.3
4	180	15888..
5	180	9147.7
6	180	6705..
7	180	9881.2
14	180	10105.5
15	180	7670.3
16	180	10239..
17	180	10195.3
18	180	10308.2

Pos	CTime	HJ DPM
19	180	34999.4
20	180	28003.5
21	180	23111.2
22	180	44245.0
23	180	44507.2
24	180	17287.5
31	180	2794.7
32	180	3078.2
33	180	733..
34	180	4136.2
35	180	4045..
36	180	3793.7

Pos	CTime	HJ DPM
37	180	23577.4
38	180	3030..2
39	180	25333.7
40	180	5256.2
41	180	6E744.1
42	180	53287.2
43	180	8519.2
44	180	9765.7
45	180	8077.1
46	180	505..7
47	180	5630.2
48	180	5130.7

Pos	CTime	HJ DPM
65	180	21746.9
66	180	15760.4
67	180	12853.2
68	180	12183.1
69	180	735..7
70	180	29437.1
71	180	592..7
72	180	6377.4
73	180	5515.4
80	180	3196.7
81	180	3445.4
82	180	3335.5

ST-9707-01 CCF-STTG1 AM404



Title	CCf-STTG1 Uptake & Inhibition with										
Column ID	A	B	C	D	E	F	G	H	I	J	K
Column Title	Control In	0.1 In	0.3 in	1.0 In	3.0 In	10.0 In	50.0 In	100.0 In	Control c	0.1 c	0.3 c
Raw or Mean	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
Mean	3.7436e+04	2.8705e+04	2.9704e+04	2.1747e+04	1.0580e+04	1.4240e+04	6.1201e+04	3.1521e+04	9133.00	8675.67	9106.00
SD	3267.41	5974.89	3933.94	0.00	4755.55	332.19	2519.70	1785.51	1288.86	768.22	937.42
Sample size	3	3	3	1	3	3	3	3	3	3	3
SEM	1886.44	3449.61	2271.26	0.00	2745.62	191.79	1454.75	1030.86	744.12	443.53	541.22
95% CI min	2.9318e+04	1.3861e+04	1.9931e+04	2.1747e+04	-1234.39	4.3415e+04	5.4941e+04	2.7085e+04	5931.03	6767.15	6777.14
95% CI max	4.5553e+04	4.3548e+04	3.9477e+04	2.1747e+04	2.2394e+04	4.5065e+04	6.7460e+04	3.5957e+04	1.2335e+04	1.0584e+04	1.1435e+04
Minimum	3.4045e+04	2.3111e+04	2.5338e+04	2.1747e+04	6705.00	4.3868e+04	5.8293e+04	2.9497e+04	7671.00	7854.00	8033.00
Maximum	4.0564e+04	3.4999e+04	3.2973e+04	2.1747e+04	1.5887e+04	1.4507e+04	6.2744e+04	3.2873e+04	1.0105e+04	9376.00	9766.00

L	M	N	O	P
1.0 c	3.0 c	10.0 c	50.0 c	100.0 c
Raw Data	Raw Data	Raw Data	Raw Data	Raw Data
6291.00	1.0247e+04	4014.67	5299.67	3326.67
736.77	56.43	202.21	290.77	123.27
3	3	3	3	3
425.38	32.58	116.75	167.87	71.17
4460.60	1.0107e+04	3512.30	4577.30	3020.43
8121.40	1.0387e+04	4517.03	6022.03	3632.90
5515.00	1.0195e+04	3799.00	5087.00	3199.00
6981.00	1.0307e+04	4200.00	5631.00	3445.00

020000

-01

ST-9707- CCF-STTG1 AM404

	1	2	3	4	5	6	7	8	9
	Concentration	Initial	In the cell	SEM	Corr fac	In the cell c	SEM c	% Control	SEM %
1	0.01	37436	9133	744	1	9133	744	100	
2	0.1	28705	8676	443	1.304	11313.504	577.672	124	1.36
3	0.3	29704	9106	541	1.26	11473.56	681.66	126	7.47
4	1	21747	6291	425	1.721	10826.811	731.425	119	8
5	3					0	0		
6	10	44240	4015	117	0.846	3396.69	98.982	37	1.084
7	50	61201	5300	168	0.612	3243.6	102.816	36	1.109

P1 127637

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THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

August 30, 1999

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/088,568*
FILING DATE: *June 09, 1998*
PCT APPLICATION NUMBER: *PCT/US99/12900*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

R. BLAKENEY
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17 1(a) OR (b)

JC514 U.S. PTO
06/60/90

A/prov

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	UCON/140/US	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Makriyannis	Alexandros		Watertown, MA, USA
Lin	Sonyuan		Storrs, CT, USA

TITLE OF THE INVENTION (280 characters max)

Anandamide Transporter Inhibitor Medications

CORRESPONDENCE ADDRESS

James E. Alix, Esq.
Alix, Yale & Ristas, LLP
750 Main Street
Hartford

CUSTOMER NO. 002543

STATE	CT	ZIP CODE	06103-2721	COUNTRY	U.S.A.

ENCLOSED APPLICATION PARTS (check all that apply)

X Specification — Number of Pages 8
— Drawing(s) — Number of Pages
X Small Entity Statement
— Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

X A check or money order is enclosed to cover the filing fees
X The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 16-2563

FILING FEE AMOUNT ($): $ 150

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government

X No
— Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE James E. Alix Date June 9, 1998

TYPED or PRINTED NAME James E. Alix REGISTRATION NO. 20,736

— Additional inventors are being named on separately numbered sheets attached hereto.

EXPRESS MAIL mailing label number EL 052 086 112 US

Date of Deposit June 9, 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Rose A. Smollen

Rose A. Smollen

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231.

Anandamide Transporter Inhibitor Medications

INVENTION DISCLOSURE Page 1 Disclosure No._____

(1) COMPLETE DESCRIPTION OF THE INVENTION: Use additional pages, if necessary, and attach any relevant sketches, diagrams, drawings, photographs or other illustrative material. ALL ATTACHED MATERIALS MUST BE SIGNED AND DATED BY EACH INVENTOR AND WITNESSED. Description may be by reference to a separate document such as a publication, manuscript, preprint or report. Such documents must be attached.

A carrier protein that transports extracellular anandamide across the cell membrane has been shown to be present in rat neurons and astrocytes. This carrier protein or anandamide transporter is believed to be responsible for the inactivation of anandamide, an endogenous cannabinoid for central cannabinoid receptors. Thus, anandamide released from neurons on depolarization is rapidly transported back into the cells and subsequently hydrolyzed by an amidase thereby terminating its biological actions. Anandamide transporter is a potential therapeutic target for the development of useful medications.

We have discovered a phenolic analog of anandamide namely N-(4-hydroxyphenyl)arachidonylamide (AM404) which inhibits the transport of anandamide across the cell membranes. AM404 does not activate cannabinoid receptors or inhibit anandamide hydrolysis *per se*. However, it does potentiate receptor-mediated anandamide responses by preventing anandamide reuptake. .....Continued on Supplement Page

(2) NOVEL FEATURES: Clearly specify the novel aspects of your invention. Compared to present technology, how is your invention different?

AM404 is a potent inhibitor of anandamide transport and it is the only compound known todate that competitively inhibits anandamide reuptake.

What deficiency in the present technology does your invention improve upon? Is it more effective? cheaper? superior in other ways?

Present cannabinoid drugs are targeted towards cannabinoid receptors (CB1 and CB2) and anandamide amidase enzyme. AM404 described in this invention targets a novel protein called anandamide transporter.

(3) STAGE OF DEVELOPMENT: Cite your specific results to date demonstrating that your concept is valid. Has your work included laboratory studies? Pilot-scale experiments? Construction and testing of a prototype?

AM404 inhibited accumulation of anandamide in rat neurons and astrocytes with an IC_{50} of 1μM for neurons and 5μM for astrocytes. In addition, AM404 potentiated and prolonged receptor-mediated effects of anandamide such as vasodialation. These experiments further support that AM404 is an inhibitor of anandamide transport.

Inventor(s) 1. _____ Date 5/12/98 Disclosed to and Understood by:

2. _____ Date 5/12/98 _____ Date 5/12/98

3. _____ Date _____ _____ Date 5/12/98

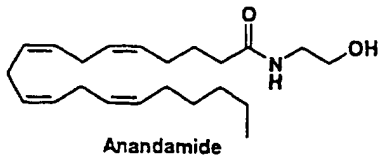
SUPPLEMENT PAGE

INVENTION DISCLOSURE FORM

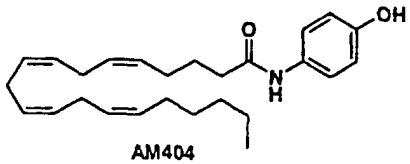
Disclosure No. _____

Continued from page 1, item 1:

Structural formulas for AM404 and anandamide are shown below.



Anandamide



AM404

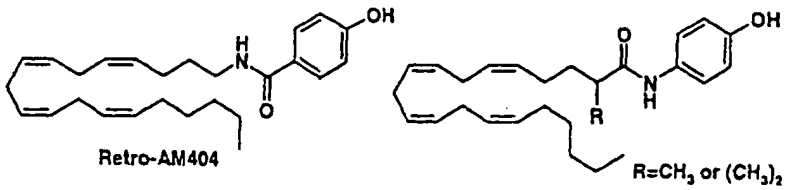
AM404 and its analogs are potential drug candidates for the treatment of ailments related to the cannabinoid system. Potential therapeutic uses of AM404 are pain alleviation (analgesia), treatment of cardiovascular diseases and blood pressure disorders.

Inventor(s) 1. A Makriyannis Date 5/12/98 Disclosed to and Understood by:

2. [signature] Date 5/12/98 [signature] Date 5/12/98

3. _____ Date _____ [signature] Date 5/12/98

(4) VARIATIONS OF THE INVENTION: Discuss all alternate forms that you can foresee for this invention, whether or not you have evaluated them to date. (For example, chemical inventions should consider analogs and derivatives.)



AM404 was first synthesized in March 1993 and tested in July 1997 as anandamide transport inhibitor

(6) INVENTOR'S PUBLICATION PLANS: Please list all your publications — theses, reports, pre-prints, abstracts, papers, etc. that pertain to the invention. Include publication dates. Also, include manuscripts for publication (submitted or not), news releases, and internal publications. Enclose copies of all the above items with this disclosure.

Beltramo, M.; Stella, N.; Calignano, A.; Lin, S.; Makriyannis, A.; Piomelli, D. Functional Role of High Affinity Anandmide Transport Inhibitor, as Revealed by Selective Inhibition. Science 1997, 277, 1094. (included) - CD8

BioWorld Today, Volume 8(162), August 21, 1997.

(7) PRIOR DISCLOSURE: Please give the details (date, place and circumstances) of any oral or written disclosures of all or part of this invention. If disclosed to specific individuals, give their names. Include professional meetings and conferences. Has this invention or a product resulting from this invention been offered for sale or license? Have any samples related to this invention been distributed?

No prior disclosure

Inventor(s) 1. A. Makriyannis Date 5/12/98 Disclosed to and Understood by:

2. Shaefeen Lin Date 5/12/98 A. Makriyannis Date 5/12/98

3. _____ Date _____ Mahavir Sharma Date 5/12/98

SUPPORTING INFORMATION

(1) PRIOR KNOWLEDGE AND COMPETING RESEARCH AND DEVELOPMENT: Please list all publications and patents by the inventor or others that relate to the invention. The inventor should thoroughly search the published literature and review closely related patents. Publications by the researchers:

1) Calignano, A.; La Rana, G.; Beltramo, M.; Makriyannis, A.; Piomelli, D. Potentiation of Anandamide Hypotension by the Transport Inhibitor, AM404. *Eur. J. Pharmacol.* 1997, *337*, R1-R2. 2) Calignano, A.; La Rana, G.; Makriyannis, A.; Lin, S.; Beltramo, M.; Piomelli, D. Inhibition of Intestinal Motility by Anandamide, an Endogenous Cannabinoid. *Eur. J. Pharmacol.* 1997, *340*, R7-R8.

List any known research groups currently engaged in research and development in this area. Include both academic and industrial researchers.

None

(2)ALTERNATE TECHNOLOGY: Describe any known alternate technologies that accomplish the same or similar purposes as this invention. List companies and products that currently use these alternate technologies.

None

(3) COMMERCIAL APPLICATION OF THE INVENTION: List all products, processes, devices, equipment, etc., to which your invention could be applied or which could result directly from your invention. Can these applications be developed in the near term (within two years) or the long term (more than two years)?

Medication to alleviate pain and treatment of cardiovascular diseases.
Long term development

What firms or types of firms do you think may be interested in the invention? Why? Name companies and specific persons if possible. Especially list companies with which you have had direct contact.

Pharmaceutical and biotech companies

(4) RESEARCH AND DEVELOPMENT PLANS: What additional research is needed to complete development and testing of the invention? Are you actively pursuing the needed work? Under whose sponsorship? About how long will this work take? What additional research support, if any, is needed for these efforts?

IDENTIFICATION AND FUNCTIONAL ROLE OF HIGH-AFFINITY ANANDAMIDE TRANSPORT

M. Beltramo*, N. Stella*, A. Calignano#, S.Y. Lin+, A. Makriyannis+ and D. Piomelli*. *The Neurosci. Inst. San Diego, CA 92121, #Sch.of Pharm. Univ. of Naples, Italy 80131, and +Sch. of Pharm. Univ. of Connecticut, Storrs, CT 06269.

Anandamide (arachidonylethanolamide) is an endogenous lipid that activates brain cannabinoid receptors. Two main pathways have been proposed for anandamide inactivation: cellular uptake and enzymatic degradation. In the present study we identified and characterized pharmacologically a high affinity anandamide uptake system in neurons and astrocytes. Exogenous [^{3}H]anandamide (spec. rad.: 221 Ci/mmol) is rapidly cleared ($t_{1/2}$=4 minutes) from the media of neurons or astrocytes in cell culture through a saturable, temperature-dependent and sodium-independent transport system. This uptake displays high affinity for [^{3}H]anandamide (neurons: Km 1.2 microM; astrocytes: Km 0.32 microM). Competition experiments with fatty acid derivatives, arachidonic acid, or palmitoylethanolamide proved its specificity. Screening of lipid uptake blockers and anandamide analogs led to the identification of a compound N-(4-hydroxyphenyl) arachidonylamide (AM404) which is potent and specific in inhibiting anandamide transport, but does not activate CB1 cannabinoid receptors and does not inhibit anandamide degradation. In cultures of cortical neurons, concentrations of anandamide higher than 0.3 microM are necessary to activate CB1 cannabinoid receptors and to revert forskolin-induced adenylyl cyclase activity. In the presence of AM404 (10 microM) the potency of anandamide is greatly increased. By contrast, AM404 has no effect on adenylyl cyclase activity when applied alone (10 microM), and does not potentiate adenylyl cyclase activity inhibition elicited by the CB1 receptor agonist WIN-55212-2 (100 nM) or by glutamate (3 microM). The hot-plate model of analgesia in the mouse was used to test the functional role of anandamide transport *in vivo*. Intravenous (i.v) administration of anandamide (20 mg/kg) induces a modest, but significant, analgesia which disappears 60 minutes after the injection and is prevented by SR-141716 (1 mg/kg, i.p.). Administration of AM404 (10 mg/kg, i.v.) has no antinociceptive effect *per se* within 60 minutes of injection, but significantly enhances and prolongs anandamide-induced analgesia. The identification in neural cells of a high-affinity [^{3}H]anandamide transport system and the discovery of selective transport blockers should be important to understand the physiological role of the endogenous cannabinoid system. In light of the multiple behavioral effects of cannabinoid receptor activation, these inhibitors might also open novel therapeutic avenues for the treatment of psychiatric and neurological disorders.

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Functional Role of High-Affinity Anandamide Transport, as Revealed by Selective Inhibition

M. Beltramo, N. Stella, A. Calignano, S. Y. Lin, A. Makriyannis, D. Piomelli*

Anandamide, an endogenous ligand for central cannabinoid receptors, is released from neurons on depolarization and rapidly inactivated. Anandamide inactivation is not completely understood, but it may occur by transport into cells or by enzymatic hydrolysis. The compound *N*-(4-hydroxyphenyl)arachidonylamide (AM404) was shown to inhibit high-affinity anandamide accumulation in rat neurons and astrocytes in vitro, an indication that this accumulation resulted from carrier-mediated transport. Although AM404 did not activate cannabinoid receptors or inhibit anandamide hydrolysis, it enhanced receptor-mediated anandamide responses in vitro and in vivo. The data indicate that carrier-mediated transport may be essential for termination of the biological effects of anandamide, and may represent a potential drug target.

Anandamide (arachidonylethanolamide) is an endogenous lipid that activates brain cannabinoid receptors and mimics the pharmacological effects of Δ^9-tetrahydrocannabinol, the active principle of hashish and marijuana (*1*). In humans, such effects include euphoria, calmness, dream states, and drowsiness (*2*). Depolarized neurons release anandamide (*3*) through a mechanism that may require the calcium-dependent cleavage of a phospholipid precursor in neuronal membranes (*4*). Like other modulatory substances, extracellular anandamide is thought to be rapidly inactivated, but the exact nature of this inactivating process is still unclear. A possible pathway is hydrolysis to arachidonic acid and ethanolamine, catalyzed by a membrane-bound fatty acid amide hydrolase (FAAH) highly expressed in rat brain and liver (*5*). Nevertheless, the low FAAH activity found in brain plasma membranes indicates that this enzyme may be intracellular (*5*), a possibility that is further supported by sequence analysis of rat FAAH (*6*). Although anandamide could gain access to FAAH by passive diffusion, the transfer rate is expected to be low because of the molecular size of this lipid mediator (*7*). In that other lipids including polyunsaturated fatty acids and prostaglandin E_2 (PGE_2) enter cells by carrier-mediated transport (*8, 9*), it is possible that anandamide uses a similar mechanism. Indeed, the existence of a rapid, saturable process of anandamide accumulation into neural cells has been reported (*3*). This accumulation may result from the activity of a transmembrane carrier, which may thus participate in termination of the biological actions of anandamide. Accordingly, we developed drug inhibitors of anandamide transport and investigated their pharmacological properties in cultures of rat cortical neurons or astrocytes.

The accumulation of exogenous [^{3}H]anandamide by neurons or astrocytes fulfills several criteria of a carrier-mediated transport (Fig. 1) (*10*). It is a rapid process that reaches 50% of its maximum within about 4 min (Fig. 1A). Furthermore, [^{3}H]anandamide accumulation is temperature-dependent (Fig 1A) and saturable (Fig. 1, B and C). Kinetic analyses revealed that accumulation in neurons can be represented by two components of differing affinities (lower affinity: Michaelis constant, K_m, = 1 2 μM, maximum accumulation rate, V_{max}, = 90.9 pmol/min per milligram of protein; higher affinity: K_m = 0.032 μM, V_{max} = 5 9 pmol/min per milligram of protein) (Fig. 1B). The higher affinity component may reflect a binding site, however, as it is displaced by the cannabinoid receptor antagonist, SR-141716-A (100 nM) (*11*). In astrocytes, [^{3}H]anandamide accumulation is represented by a single high-affinity component (K_m = 0.32 μM, V_{max} = 171 pmol/min per milligram of protein) (Fig. 1C). Such apparent K_m values are similar to those of known neurotransmitter uptake systems (*12*) and are suggestive therefore of high-affinity carrier-mediated transport.

To characterize further this putative anandamide transport, we used cortical astrocytes in culture As expected from a selective process, the temperature-sensitive component of [^{3}H]anandamide accumulation was prevented by nonradioactive anandamide, but not by palmitoylethanolamide, arachidonate, prostanoids, or leukotrienes (Fig. 2A). Replacement of extracellular Na$^+$ with *N*-dimethylglucosamine or choline had no effect (as percentage of control: *N*-dimethylglucosamine, 124 ± 12%; choline, 98 ± 14%; mean ± SEM, n = 6), suggesting that [^{3}H]anandamide accumulation is mediated by a Na$^+$-independent mechanism, which has been observed with other lipids (*8, 9*). Moreover, inhibition of FAAH activity by treating the cells with (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (25 μM) or linoleyl trifluoromethyl ketone (15 μM) (*13, 14*) had no effect (Fig. 2, B and C) This indicates that anandamide hydrolysis did not provide the driving force for anandamide transport into astrocytes within the

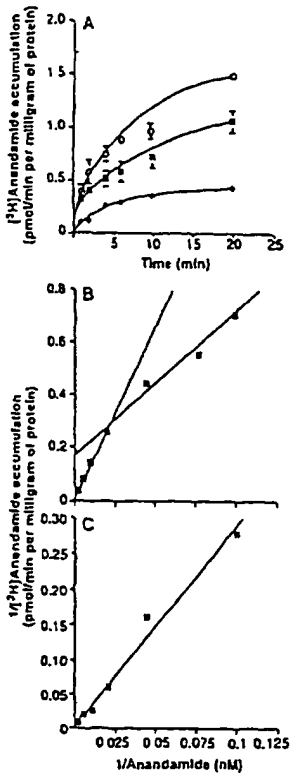

Fig. 1. (A) Time course of [^{3}H]anandamide accumulation in rat cortical neurons (circles) or astrocytes (squares) at 37°C, and astrocytes at 0° to 4°C (diamonds). Results are expressed as mean ± SEM of 6 to 12 independent determinations. (B and C) Lineweaver-Burk analyses of [^{3}H]anandamide accumulation (37°C, 4 min) in neurons (B) or astrocytes (C). Results are from one experiment representative of three performed in duplicate with each cell type. The [^{3}H]anandamide accumulation assay has been described (*10*).

M. Beltramo, N. Stella, D. Piomelli, The Neurosciences Institute, 10640 J. J. Hopkins Drive, San Diego, CA 92121, USA.
A. Calignano, School of Pharmacy, University of Naples, Italy 80131.
S. Y. Lin and A. Makriyannis, School of Pharmacy, University of Connecticut, Storrs, CT 06269, USA.
*To whom correspondence should be addressed. E-mail: pomel@nsi.edu

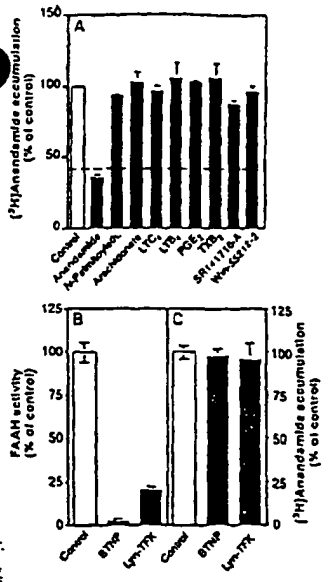

Fig. 2. (A) Selectivity of [³H]anandamide accumulation in cortical astrocytes. Accumulation was measured after a 4-min incubation with [³H]anandamide at 37°C, in the absence (control) or presence of nonradioactive anandamide (100 μM), *N*-palmitoylethanolamide (100 μM), arachidonate (100 μM), leukotriene C_4 (LTC_4; 1 μM), leukotriene B_4 (LTB_4; 1 μM), PGE_2 (100 μM), or thromboxane B_2 (TXB_2; 50 μM). The broken line indicates nonspecific [³H]anandamide accumulation in cells measured at 0° to 4°C (43 ± 3% of total accumulation, which in these experiments was 43,104 ± 1249 dpm per well). Results are expressed as mean ± SEM (n = 6 to 9). Effects of FAAH inhibitors on (B) FAAH activity and (C) [³H]anandamide accumulation in cortical astrocytes. Cells were incubated for 10 min with (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (BTNP, 25 μM) or linoleyl trifluoro methylketone (Lyn-TFK, 15 μM), and then with the same drugs plus [³H]anandamide for an additional 20 min. The total radioactivity in cell lipid extracts (to measure [³H]anandamide transport) (*10*) and radioactivity in nonesterified arachidonate (to measure FAAH activity) (*13*) were measured separately in samples of lipid extracts prepared from the same cultures.

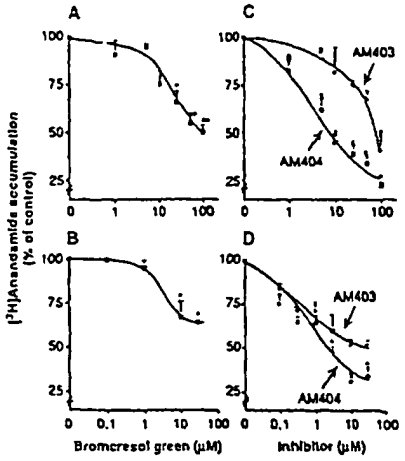

Fig. 3. Inhibition of [³H]anandamide accumulation by bromcresol green in (A) astrocytes or (B) neurons. One asterisk indicates $P < 0.05$ and two asterisks $P < 0.01$ [analysis of variance (ANOVA) followed by Bonferroni test] compared with control [³H]anandamide accumulation. Inhibition of [³H]-anandamide accumulation by AM404 (squares) or AM403 (diamonds) in (C) astrocytes or (D) neurons. The asterisk indicates $P < 0.05$ (paired Student's t test) between AM404 and AM403). In all experiments, cells were incubated with the inhibitors for 10 min before the addition of [³H]anandamide for an additional 4 min. Results are expressed as mean ± SEM of three to nine independent determinations.

time frame of our experiments. Finally, the cannabinoid receptor agonist WIN-55212-2 (1 μM) and antagonist SR-141716-A (10 μM) also had no effect, suggesting that receptor internalization was not involved (Fig. 2A).

A primary criterion for defining carrier-mediated transport is pharmacological inhibition. To identify inhibitors of anandamide transport, we first examined compounds that prevent the cellular uptake of other lipids, such as fatty acids (phloretin, 50 μM), phospholipids (verapamil, 100 μM; quinidine, 50 μM), or PGE_2 (bromcresol green, 0.1 to 100 μM) (*15*). Among the compounds tested, only bromcresol green interfered with anandamide transport, albeit with limited potency and partial efficacy (Fig. 3, A and B). Bromcresol green inhibited [³H]anandamide accumulation with an IC_{50} (concentration needed to produce half-maximal inhibition) of 4 μM in neurons and 12 μM in astrocytes and acted noncompetitively (*16*). Moreover, bromcresol green had no significant effect on the binding of [³H]WIN-55212-2 to rat cerebellar membranes (inhibition constant, K_i = 22 μM), FAAH activity in rat brain microsomes (IC_{50} > 50 μM), and uptake of [³H]arachidonate or [³H]ethanolamine in astrocytes (121 ± 13% and 103 ± 12%, respectively, at 50 μM bromcresol green, n = 3) (*17*). The sensitivity to bromcresol green, which blocks PGE_2 transport, raised the question of whether anandamide accumulation occurred by means of a PGE_2 carrier. That this is not the case was shown by the lack of [³H]PGE_2 accumulation in neurons or astrocytes (*18*) and by the inability of PGE_2 to interfere with [³H]anandamide accumulation (Fig. 2A). Previous results indicating that expression of PGE_2 transporter mRNA in brain tissue is not detectable further support this conclusion (*9*).

To search for more potent anandamide transport inhibitors, we synthesized and tested a series of structural analogs of anandamide (*19*). From this screening, we selected the compound *N*-(4-hydroxyphenyl)arachidonylamide (AM404), which was both efficacious and relatively potent (Fig. 3, C and D; IC_{50} was 1 μM in neurons and 5 μM in astrocytes). As we anticipated from its chemical structure, AM404 acted as a competitive inhibitor (*20*), suggesting that it may serve as a transport substrate or pseudosubstrate. In contrast, at the concentrations tested AM404 had no effect on FAAH activity (IC_{50} > 30 μM) or on uptake of [³H]arachidonate or [³H]ethanolamine (102 ± 4% and 96 ± 14%, respectively, at 20 μM AM404, n = 6). Furthermore, a positional isomer of AM404, *N*-(3-hydroxyphenyl)-arachidonylamide (AM403), was significantly less effective than AM404 in inhibiting transport (Fig. 3, C and D). These data provide pharmacological evidence for the existence of a specific anandamide transporter and suggest (i) that neurons and astrocytes may act synergistically in the brain to dispose of extracellular anandamide and (ii) that the transport systems in these two cell types may differ kinetically and pharmacologically (Fig. 1, B and C, and Fig. 3, C and D).

The identification of inhibitors allowed us to examine whether transmembrane transport participates in terminating anandamide responses mediated by cannabinoid receptor activation. Cannabinoid receptors of the CB1 subtype are expressed in neurons (*21*) where they are negatively coupled to adenylyl cyclase activity (*22*). Accordingly, in cultures of rat cortical neurons the cannabinoid receptor agonist WIN-55212-2 inhibited forskolin-stimulated adenosine 3′,5′-monophosphate (cAMP) accumulation (control: 39 ± 4 pmol per milligram of protein; 3 μM forskolin: 568 ± 4 pmol per milligram of protein; forskolin plus 1 μM WIN-55212-2: 220 ± 24 pmol per milligram of protein), and this inhibition was prevented by the antagonist SR-141716-A (1 μM) (555 ± 39 pmol/mg of protein, n = 9) (*23*). Anandamide produced a similar effect, but with a potency (IC_{50} 1 μM) that was 1/20 of that expected from its binding

constant for CB1 cannabinoid receptors (K_i = 50 nM) (1) (Fig. 4A). The transport inhibitor AM404 bound to CB1 receptors with low affinity (K_i = 1.8 μM) (19) and did not reduce cAMP concentrations when applied at 10 μM (Fig. 4B). Nevertheless, the drug enhanced the effects of anandamide, increasing the potency (by a factor of 10) and decreasing the threshold (by a factor of 1/100), an effect that was prevented by SR-141716-A (Fig. 4A). Thus, a concentration of anandamide that was below threshold when applied alone (0.3 μM) produced an almost maximal effect when applied with AM404 (Fig. 4B). Bromcresol green and AM403, which were less effective than AM404 in inhibiting anandamide transport (Fig. 3), were also less effective in enhancing the anandamide response (Fig. 4B). Furthermore, the decreases in cAMP concentrations produced by WIN-55212-2 (which stimulates CB1 receptors but is not subject to physiological clearance) or glutamate [which stimulates metabotropic receptors negatively coupled to adenylyl cyclase (24) and is cleared by a selective transporter (25)] are not affected by any of the anandamide transport inhibitors tested (26).

These results suggest that pharmacological blockade of carrier-mediated transport protects anandamide from physiological inactivation, enhancing the potency of anandamide to nearly that expected from its affinity for CB1 cannabinoid receptors in vitro. To find out whether this potentiation occurs in vivo, we tested the effects of AM404 on the antinociceptive activity of anandamide in mice. Intravenous anandamide (20 mg per kilogram of body weight) elicited a modest but significant analgesia, as measured by the hot plate test (27) ($P < 0.05$, Student's t test), this analgesia disappeared 60 min after injection and was prevented by SR-141716-A (Fig. 4C) (28). Administration of AM404 (10 mg/kg, intravenously) had no antinociceptive effect within 60 min of injection but significantly enhanced and prolonged anandamide-induced analgesia (Fig. 4C) ($P < 0.01$, Student's t test).

Our findings indicate that a high-affinity transport system present in neurons and astrocytes has a role in anandamide inactivation by removing this lipid mediator from the extracellular space and delivering it to intracellular metabolizing enzymes such as FAAH (5, 6). Therefore, the identification of selective inhibitors of anandamide transport should be instrumental in understanding the physiological roles of the endogenous cannabinoid system and may lead to the development of therapeutic agents.

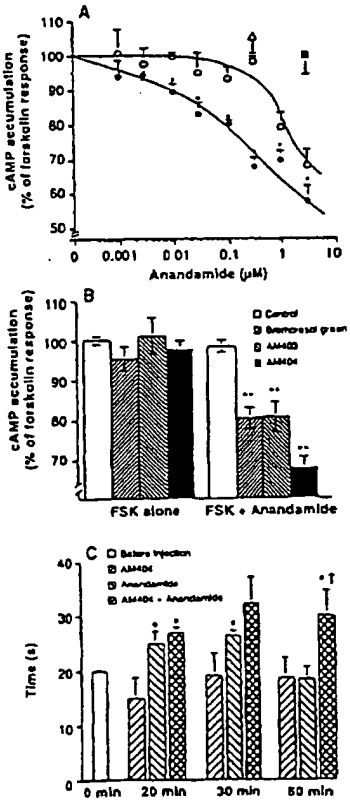

Fig. 4. (A) Effects of AM404 on anandamide-induced inhibition of adenylyl cyclase activity in cortical neurons. The neurons were stimulated with forskolin (3 μM) in the presence of anandamide (0.001 to 3 μM; open circles), anandamide (0.001 to 3 μM) plus AM404 (10 μM) (filled circles), anandamide (3 μM) plus SR-141716-A (1 μM) (square), or anandamide (0.3 μM) plus AM404 (10 μM) and SR-141716-A (1 μM) (triangle). (B) Effects of anandamide transport inhibitors on anandamide-induced inhibition of adenylyl cyclase activity. Forskolin (FSK)-stimulated neurons were incubated with AM404, AM403, or bromcresol green (each at 10 μM) without (FSK alone) or with (FSK + anandamide) 0.3 μM anandamide. Results are expressed as mean ± SEM of nine independent determinations. One asterisk indicates $P < 0.05$ and two asterisks $P < 0.01$ (ANOVA followed by Bonferroni test). (C) Effects of AM404 on the analgesic activity of anandamide in the hot plate test. Three groups of six mice received AM404 (10 mg/kg, intravenous), anandamide (20 mg/kg, intravenous), or anandamide plus AM404. The hot plate test (55.5°C) was performed at the times indicated, and latency to jump (in seconds) was measured before (control) and after the drugs were injected. In all groups, latency to jump before injections was 21 ± 0.6 s (n = 18). A fourth group of mice received injections of vehicle alone (saline containing 20% dimethyl sulfoxide), which did not affect latency to jump. One asterisk indicates $P < 0.05$ compared with uninjected controls (ANOVA followed by Bonferroni test), and one cross indicates $P < 0.01$ compared with anandamide-treated animals (Student's t test).

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10. Cultures of cortical neurons [N. Stella, L. Pellerin, P. Magistretti, *J. Neurosci.* 15, 3307 (1995)] or astrocytes (13) were prepared from rat embryos and were used after 4 to 6 days and 21 to 25 days in vitro, respectively. Accumulation of [^{3}H]anandamide (221 Ci/mmol, New England Nuclear, Wilmington, DE) was measured by incubating the cells (six-well plates) for various times in Krebs buffer [136 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, and 20 mM Tris/Hepes base; (pH 7.4), at 37°C] containing [^{3}H]anandamide (0.45 nM, brought to 100 nM with nonradioactive anandamide). Incubations were stopped by aspirating the media, and cells were rinsed with Krebs buffer containing bovine serum albumin (BSA, 0.1% w/v) and subjected to extraction with methanol and chloroform. Radioactivity in the extracts was measured directly or after fractionation of cell lipids by thin-layer chromatography (13). For kinetic analyses, the neurons were incubated for 4 min at 37°C in the presence of 10 to 500 nM anandamide containing 0.05 to 2.5 nM [^{3}H]anandamide. We subtracted nonspecific accumulation (measured at 0° to 4°C) before determining kinetic constants by Lineweaver-Burk analysis.
11. M. Beltramo and D. Piomelli, unpublished data.
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16. In astrocytes, V_{max} values for [^{3}H]anandamide accumulation were 200 pmol/min per milligram of protein without bromcresol green, and 111 pmol/min per milligram of protein with bromcresol green (10 μM). Apparent K_m values were 0.24 and 0.25 μM, respectively (n = 6).
17. Displacement of [^{3}H]WIN-55212-2 binding (40 to 60 Ci/mmol; New England Nuclear) to rat cerebellar

membranes (0.1 mg/ml) was determined as described [J. E. Kuster *et al.*, *J. Pharmacol Exp Ther* 264, 1352 (1993)]. Nonspecific binding was measured in the presence of 1 μM nonradioactive WIN-55212-2. FAAH activity was measured in rat brain particulate fractions as described (13). The uptake of [³H]arachidonate (Amersham, 200 Ci/mmol, 5 nM brought to 100 nM) and [³H]ethanolamine (Amersham, 50 Ci/mmol; 20 nM brought to 100 nM) was determined on cortical astrocytes for 4 min as described (10). The control uptake for [³H]arachidonate was 16729 ± 817 dpm per well and for [³H]ethanolamine it was 644 ± 100 dpm per well ($n = 6$).

18. Neurons or astrocytes were incubated for 4 min at 37°C in Krebs buffer containing [³H]PGE_2 (0.67 nM brought to 100 nM with nonradioactive PGE_2, 171 Ci/mmol, New England Nuclear). After rinsing with Krebs buffer containing BSA, we subjected the cells to lipid extraction and counted radioactivity in the extracts. On average, neurons contained 245 ± 65 dpm per well and astrocytes 302 ± 20 dpm per well; nonspecific accumulation in astrocytes at 0° to 4°C was 355 ± 28 dpm per well ($n = 6$).
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20. In astrocytes, apparent K_m values for [³H]anandamide accumulation were 0.11 μM without AM404 and 0.27 μM with AM404 (10 μM). V_{max} values were 29 pmol/min per milligram of protein without AM404 and 26 pmol/min per milligram of protein with AM404, respectively ($n = 6$).
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23. Cortical neurons were prepared in 12-well plates and used after 4 to 6 days in vitro. Incubations were carried out in the presence of forskolin (3 μM) and isobutyl methyl xanthine (1 mM). The cAMP concentrations were measured by radioimmunoassay with a commercial kit (Amersham, Arlington, IL) and following manufacturer's instructions.
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26. The amounts of cAMP in the presence of a concentration of WIN-55212-2 below threshold (1 nM, determined in preliminary experiments) were 96.7 ± 2.5% of forskolin alone and were not significantly affected by 10 μM AM404 (89.8 ± 2.6%), 10 μM AM403 (92.4 ± 2.3%), or 10 μM bromocresol green (92.9 ± 2.3%) ($n = 3$). In the presence of a concentration of glutamate below threshold (3 μM) (24), cAMP concentrations were 91.6 ± 2% of forskolin alone and were not significantly affected by AM404 (84.4 ± 4.9%), AM403 (89.5 ± 2.4%), or bromocresol green (84.4 ± 3%) ($n = 3$).
27. E. Fride and R. Mechoulam, *Eur. J. Pharmacol.* 231, 313 (1993); P. B. Smith *et al.*, *J. Pharmacol Exp Ther.* 270, 219 (1994).
28. The hot plate test (55.5°C) was carried out on male Swiss mice (25 to 30 g, Nossan, Italy) following standard procedures (F. Porreca, H. L. Mosberg, R. Hurst, V. J. Hruby, T. F. Burks, *J. Pharmacol. Exp. Ther.* 230, 341 (1984)). Anandamide and AM404 were dissolved in 0.9% NaCl solution containing 20% dimethyl sulfoxide and injected intravenously at 20 mg/kg and 10 mg/kg, respectively. To determine whether cannabinoid receptors participate in the effect of anandamide, we administered anandamide (20 mg/kg intravenously) or anandamide plus SR141716-A (2 mg/kg, subcutaneously) to two groups of six mice each. In mice that received anandamide alone, latency to jump increased from 21.7 ± 1.5 s to 30.7 ± 0.8 s ($P < 0.05$, ANOVA) 20 min after injection. In contrast, in mice that received anandamide plus SR141716-A, the latency to jump was not affected (19.6 ± 3.1 s).
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